RESEARCH ARTICLE

In vitro cell-based assay for activity analysis of staphylococcal enterotoxin A in food

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Abstract

Staphylococcal enterotoxins (SEs) are a leading cause of food poisoning and have two separate biological activities; it causes gastroenteritis and functions as a superantigen that activates large numbers of T cells. *In vivo* monkey or kitten bioassays were developed for analysis of SEs emetic activity. To overcome the inherent limitations of such bioassays, this study describes an *in vitro* splenocyte proliferation assay based on SEs superantigen activity as an alternative method for measuring the activity of staphylococcal enterotoxin A (SEA). After incubation of splenocytes with SEA, cell proliferation was measured by labeling the proliferating cells' DNA with bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) and quantifying the incorporated BrdU by immunohistochemistry. BrdU labeling is shown to be highly correlated with SEA concentration ($R^2 = 0.99$) and can detect 20 pg mL^{-1} of SEA, which is far more sensitive than most enzyme-linked immunosorbent assays. Our assay can also distinguish between active toxin and inactive forms of the toxin in milk. By applying immunomagnetic beads that capture and concentrate the toxin, our assay was able to overcome matrix interference. These results suggest that our *in vitro* cell-based assay is an advantageous practical alternative to the *in vivo* monkey or kitten bioassays for measuring SEA and possibly other SEs activity in food.

Introduction

Staphylococcus aureus is one of the major bacterial pathogens causing clinical infection and food-borne illnesses, affecting a large number of people throughout the world (Dinges *et al.*, 2000). This bacterium produces a group of 21 (known) staphylococcal enterotoxins (SEs) implicated in several illnesses including food-borne diseases resulting from consumption of a broad variety of contaminated food (e.g. meats, dairy products, salads and baked goods) (Michanie *et al.*, 1988; Isigidi *et al.*, 1992; Asao *et al.*, 2003; Ikeda *et al.*, 2005a, b). Gastrointestinal symptoms consist of vomiting, nausea and diarrhea. In addition, SEs have also been implicated in other diseases such as atopic eczema, (Bunikowski *et al.*, 1999; Herz *et al.*, 1999; Mempel *et al.*, 2003) rheumatoid arthritis (Howell *et al.*, 1991; Bunning *et al.*, 1997) and urticaria (Ye *et al.*, 2008), and staphylococcal enterotoxin B (SEB) is recognized as a potential bioweapon (Henghold, 2004). The importance of SEs can be demonstrated by a recent contamination of powdered skim milk with SEA causing an extensive outbreak (13 420 cases) in Japan (Asao *et al.*, 2003; Ikeda *et al.*, 2005a, b). This outbreak emphasizes the need to develop better methods to detect active SEs.

SEs have two separate biological activities: they act on gastrointestinal tract, causing gastroenteritis and act as a superantigen on the immune system. Functional enterotoxin binds to the α-helical regions of the major histocompatibility complex class II molecules outside the peptide binding groove of the antigen-presenting cells (APC), and most SEs, except SEH, also bind to the variable region (V-β) on the T-cell receptor. The toxin then forms a bridge between T cells and APC, causing activation and proliferation of a large number (*c.* 20%) of T cells. Because previous research has shown that emetic activites and superantigenic activities of SEs are related (Hui *et al.*, 2008; Hu *et al.*, 2009) we utilized the mechanism superantigens have on T cells to develop a cell-based assay that distinguishes between active toxin and inactive toxin.

The current test to detect active SEs is an *in vivo* monkey or kitten bioassay (Bergdoll, 1988; Bennett, 2005). This activity test involves intravenous administration of the toxin into kittens or administrating a 25–100-mL food sample by gavage into the rhesus monkey's stomach and observing vomiting reaction. On administration of 10 mg, vomiting occurs only with 50% of the animals. This procedure has low sensitivity and poor reproducibility, requires many animals and raises ethical concerns with regard to the use of experimental animals. Enzyme-linked immunosorbent assays (ELISA) and MS have been developed for several SEs (Bennett, 2005; Dupuis *et al.*, 2008) with typical detection of 1 ng mL^{-1}. Other immunological methods for SEs detection were developed, including several biosensors (Rasooly & Herold, 2006). However, these immunological methods cannot distinguish between active and inactive toxin, and antibodies can react with a food sample and give false-positive results (Park *et al.*, 1992). The main objective of this study was to develop a nonradioactive cell-based assay for detection of active SEA in food. The assay described in this study utilized immunomagnetic beads that identify and concentrate specific SEs, by exploiting the superantigen activity of SEA that activates large numbers of T cells. We were able to develop a cell-based assay that is more sensitive than the *in vivo* monkey or kitten bioassay.

Materials and methods

Chemicals and reagents

SEA and purified anti-SEA antibody was obtained from toxin technology (Sarasota, FL).

Nonfat dry milk was obtained from Nestle Carnation (Vevey, Switzerland).

Splenocyte isolation

Spleen from C57BL/6 female mice were aseptically removed and disrupted using a syringe and needle in Russ-10 cell culture medium [made by combining 450 mL of RPMI 1640 medium without glutamine (Gibco), 50 mL fetal bovine serum (Hyclone, Logan, UT), 5 mL 200 mM glutamine (Gibco), 5 mL antibiotic–antimycotic (Gibco; containing penicillin, streptomycin and fungizone), 5 mL nonessential amino acid mix (Gibco), 5 mL sodium pyruvate (Gibco) and 0.25 mL of 100 mM β-mercaptoethanol (Sigma)]. Cells were centrifuged at 200 ***g*** at 4 °C for 10 min. Red blood cells were then lysed by adding red cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA). Cells were again centrifuged and resuspended in Russ-10 medium, and viable cells were counted using trypan blue and a hemocytometer.

Activity assay for SEA

Cells were placed in 96-well plates (1×10^6 mL^{-1}, 0.2 mL) in Russ-10 medium and treated with various concentrations of SEA ranging from 400 ng mL^{-1} to 4 pg mL^{-1} following incubation at 37 °C in a 5% CO_2 incubator. After incubation for 48 h, cell proliferation was measured by adding bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU), which was incorporated into DNA of dividing cells, 4 h before fixation as described by the manufacturer (Calbiochem, San Diego, CA). Spectroscopic measurements were made at $OD_{620\,nm}$ and $OD_{450\,nm}$.

SEA magnetic beads preparation

One hundred microliters of Dynabeads M-280 tosylactivated (Invitrogen, Carlsbad, CA) were washed twice with 600 μL of 0.1 M sodium borate buffer, pH 9.5, and diluted in the same buffer to 2×10^9 beads mL^{-1}. Purified anti-SEA antibody (30 μg) was added to 1×10^8 beads (50 μL). The antibody and beads were incubated for 24 h at 37 °C on a slow shaker to facilitate covalent binding. The coated beads were washed twice for 5 min at 4 °C with 1 mL phosphate-buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA), washed once for 4 h at 37 °C with 0.2 M Tris-HCl, pH 8.5, containing 0.1% BSA, and washed once more for 5 min at 4 °C with PBS, pH 7.4, containing 0.1% BSA. The beads were resuspended in 50 μL of the Tris-BSA buffer.

Sample preparation of food

Preserved beef paste or chicken (71 g; Gerber) was added to 90 mL of PBS buffer, vortexed and centrifuged at 3200 ***g*** for 5 min at 4 °C. The supernatant below the layer of fat was transferred to a new tube and spiked with 100, 50, 10 and 5 ng mL^{-1} of SEA. Milk was prepared with 5% nonfat dry milk dissolved in water (Nestle, Solon, Ohio).

Sample binding and disassociation of SEA from beads

Fifteen microliters of the immunomagnetic beads were incubated with a tilting motion at 4 °C with 4 mL of spiked milk, chicken or beef supernatant. After 24 h, the tube was placed on a magnet for 2 min to collect the beads. The beads were washed twice with PBS, pH 7.4, containing 0.1% BSA. Toxin was eluted with 7.5 μL of 100 mM glycine-HCl (pH 2.5) then neutralized with 7.5 μL of 2 × Tris-buffered saline (TBS) (pH 8.3).

Statistical analysis

Statistical analysis was performed using SIGMASTAT 3.5 for Windows (Systat Software, San Jose, CA). Multiple

comparisons of the spiked food items were made. One-way ANOVA was used to compare control unspiked food with food that contained increasing concentrations of SEA in the first experiment. The experiments were repeated at least three times and results with $P < 0.05$ were considered statistically significant.

Results

Quantitative cell-based assay for measuring biologically active SEA

To evaluate the ability of the *in vitro* cell-based assay to quantify SEA, we measured the effect of various concentrations of SEA, ranging from 10 ng mL^{-1} to 10 pg mL^{-1}, on splenocyte proliferation. Newly synthesized DNA was measured by adding BrdU (an indication of cell proliferation), followed by spectroscopic measurement. As shown in Fig. 1 there is a linear correlation ($R^2 = 0.99$) between concentration of SEA and the amount of newly synthesized DNA measured by BrdU. In terms of sensitivity, this cell-based assay enables the detection of 10 pg mL^{-1} of SEA, an amount which is far less than a typical ELISA assay of 1 ng mL^{-1}.

Activity assay for SEA in milk

To optimize the sensitivity of the assay, it is essential to study the effects of food matrices on cell viability and cell mitogenic activity. Increasing volumes of spiked milk or water (10 ng mL^{-1}) was added to aliquots of splenocyte suspension (10^6 mL^{-1}). After incubation for 2 days newly synthesized DNA was measured. Our results (Fig. 2) show that incubation of the cell culture with milk concentrations of 5%, 10% and 20% decreased viable cell count by 49%, 75% and 85%, respectively, and inhibited mitogenic activity of SEA, compared with dilution of cell culture with water.

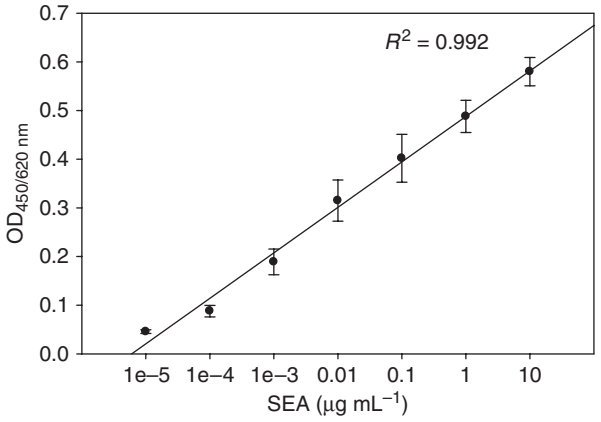

Fig. 1. Detection of SEA in cell culture medium. Cells were spiked with increasing concentrations of SEA. After incubation for 2 days newly synthesized DNA was measured. Error bars represent SEs.

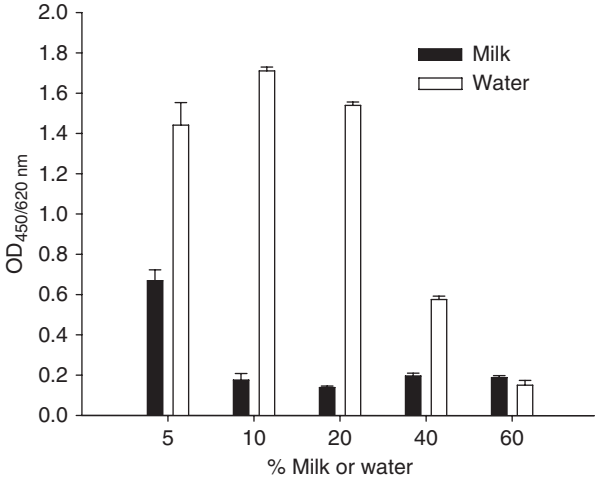

Fig. 2. Food matrix decreases newly synthesized DNA. Splenocytes were diluted with various volumes of spiked milk or water. After incubation for 2 days newly synthesized DNA was measured. Error bars represent SEs.

These results demonstrate that it is the milk matrix interference and not just the dilution of the culture medium that reduces both splenocyte cell viability and SEA mitogenic activity. Therefore, it is likely that application of this assay to complex food samples will require either dilution of the matrix to $< 5\%$ or concentration and purification of SEA before addition to the cells.

To reduce food matrix interference, 5 μL (2.5%) of milk spiked with different concentrations of SEA (25–400 ng mL^{-1}) were added to 195 μL of cell culture. As shown in Fig. 3, at this dilution rate SEA can be assayed and correlated between SEA concentration and cell proliferation measured by newly synthesized DNA.

The effect of matrix on heat inactivation of SEA

Milk is routinely heat treated; such heating changes the milk protein's conformation, solubility and other protein characteristics all effect protein–protein interaction. To determine if milk in which the toxin is present and influence toxin–protein interaction will affect the thermal inactivation of the toxin, we spiked autoclaved milk, and milk that was not autoclaved with SEA (10 ng mL^{-1}) and then heated these samples at 99 °C for various amounts of time (0–3 h). To reduce food matrix interference we diluted the cell culture to 2.5% of milk. As shown in Fig. 4, the initial mitogenic activity of SEA in autoclaved milk was lower than the unautoclaved, and this activity dropped sharply upon heating for 15 min while unautoclaved milk still showed SEA activity. The time required for complete inactivation of SEA in unautoclaved milk is seven times greater than that in autoclaved milk. SEA in unautoclaved milk was more heat resistant and retained its mitogenic activity even after treatment for 105 min. Only after 2 h SEA in unautoclaved

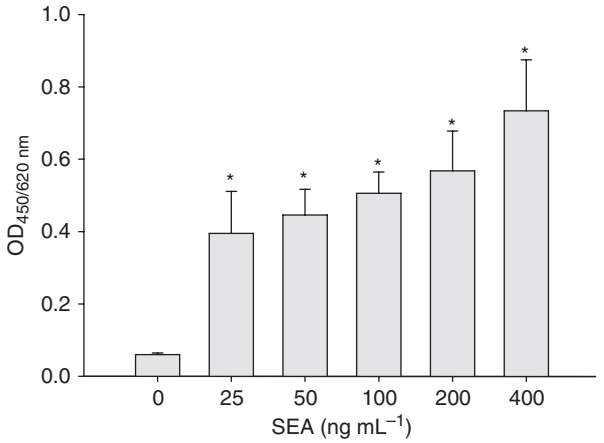

Fig. 3. Detection of SEA in milk. Milk was spiked with increasing concentrations of SEA. Five microliters of spiked milk was added to 195 μL of cell culture media; after incubation for 2 days newly synthesized DNA was measured. Error bars represent SEs, and an asterisk indicates significant differences ($P < 0.05$) between spiked and unspiked milk.

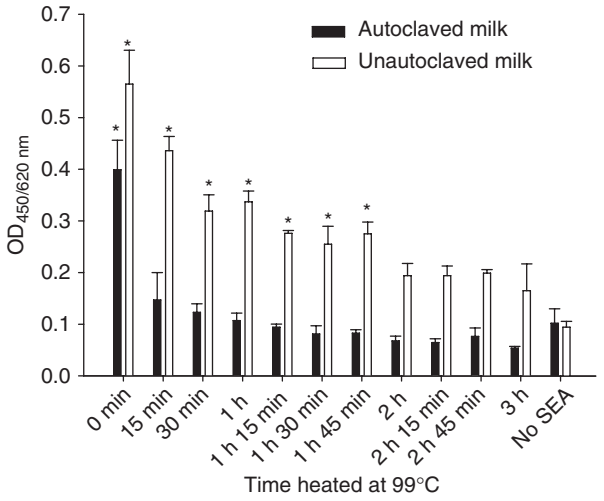

Fig. 4. Unautoclaved milk protects mitogenic activities of SEA after thermal treatment. Milk and autoclaved milk were spiked with 400 ng mL^{-1} of SEA and heated at 99 °C for various time points. After 5 μL of the milk was added and incubated with splenocytes for 2 days newly synthesized DNA was measured. Error bars represent SEs, and an asterisk indicates significant differences ($P < 0.05$) between spiked and unspiked milk.

milk lost its mitogenic activity compared with autoclaved milk in which SEA lost its mitogenic activity in < 15 min. These results demonstrate that SEA thermal inactivation is highly dependent on the medium in which the toxin is present.

Extraction and concentration of SEA from milk

To analyze larger volumes of milk (> 5 μL) and to reduce food matrix interference, shown in Fig. 2, we used immunomagnetic beads to extract and concentrate the toxin. Milk spiked with 50–200 ng mL^{-1} of SEA was incubated with immunomagnetic beads and after washing the beads multiple times (to remove milk components) the SEA-bound beads were incubated with splenocytes at a ratio of one bead to one cell and 250 beads to one cell. As shown in Fig. 5, the presence of a low ratio of beads (1 : 1) reduces cell proliferation compared with high ratio (1 : 250) especially at high concentration of SEA (200 ng mL^{-1}). Excess immunomagnetic beads per cell did not interfere with cell proliferation, on the contrary; more beads capture more toxins from the spiked milk. At ratios of 250 beads to one cell, proliferation was significantly higher in both concentrations, and the limit of detection was 50 ng mL^{-1}.

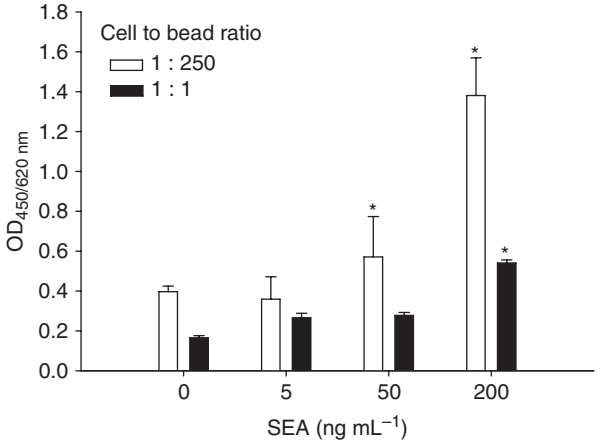

Fig. 5. Detection of SEA after extraction from spiked milk. Immunomagnetic beads were incubated with milk spiked with increasing concentrations of SEA. The washed beads were incubated with splenocytes for 2 days and newly synthesized DNA was measured. Error bars represent SEs, and an asterisk indicates significant differences ($P < 0.05$) between a ratio of 1 : 1 and 250 beads to one cell.

Disassociation of SEA from immunomagnetic beads

To further increase the sensitivity of the assay, and evaluate the ability of the assay to detect SEA in foods containing large amounts of fat and protein that have been associated with SEA outbreak, we spiked milk, chicken and beef paste with various concentrations of SEA. We disassociated SEA from immunomagnetic beads with glycine-HCl (pH 2.5) and neutralized the reaction with 2 × TBS (pH 8.3). Spiked milk or eluted toxin from beads (including autoclaved eluted sample), was added to splenocytes, and the proliferation assay was performed. As shown in Fig. 6a and b, the cell proliferated in a dose-dependent manner to the eluted toxin. As shown in Fig. 6b, there was no statistical difference between the unspiked milk and spiked milk at this low concentration (4–500 pg mL^{-1}); however, when we used the immunomagnetic beads and

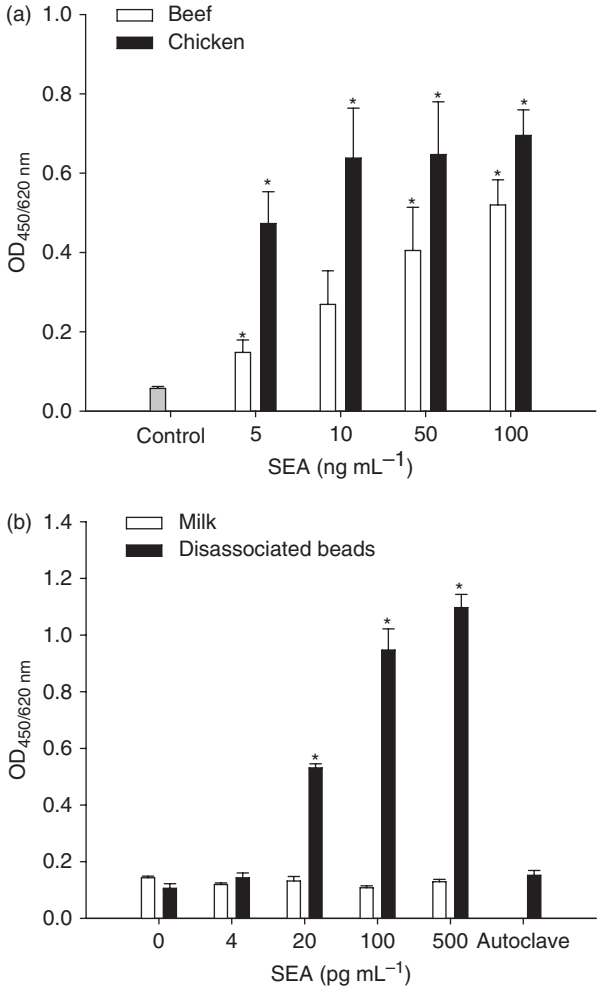

Fig. 6. Increasing the detection limit after disassociating SEA from beads. Beef and chicken (a) or milk (b) were spiked with increasing concentrations of SEA and incubated for 16 h with immunomagnetic beads. The toxin was disassociated from beads and incubated with splenocytes for 2 days. Newly synthesized DNA was measured. Error bars represent SEs, and an asterisk indicates significant differences ($P < 0.05$) between spiked and unspiked food.

released the SEA from the beads it increased the sensitivity of the assay by 2500 times from 50 ng mL^{-1} (Fig. 5) to 20 pg mL^{-1}. The newly synthesized DNA was proportional to SEA concentrations in a dose-dependent manner. When milk spiked with 500 pg mL^{-1} of toxin was heated at 121 °C for 15 min (autoclave) to deactivate the toxin there was no difference between autoclaved spiked milk and unspiked milk. This shows that our assay is able to distinguish between active and inactive forms of the toxin.

Discussion

In this study, we evaluated a potential alternative method to the *in vivo* monkey and kitten bioassays, which are currently accepted methods for measuring biological activity of SEs. While this vomiting test is very fast (2.5 h), the assay has low sensitivity, poor reproducibility and requires many animals; therefore, these assays are impractical for testing a large number of samples. In this work, we tested the most widespread SE serotype, SEA, which is associated with (78%) of staphylococcal outbreaks (Vernozy-Rozand *et al.*, 2004). We utilized the effect that SEA has on activating T-cells by incubating SEA with splenocytes and measuring cell proliferation.

Measuring emetic activities is the main issue concerning staphylococcal food poisoning. Several studies have shown that there is a relation between emetic and superantigenic activities. When an antibody was used to inhibit SEA-induced proliferation of T-cells it also significantly reduced SEA-induced vomiting in animals (Hu *et al.*, 2009). And when site-directed mutagenesis was used to inactivate the emetic activity of SEC2 it also abolished its superantigenic activities (Hui *et al.*, 2008). These works suggest that abolishing superantigenic activities may result in abolishing emetic activities. Thus, our assay that measures superantigenic activities will most likely display a relation to emetic activities.

We detected cell proliferation by adding nonradioactive analog of thymidine that incorporates into newly synthesized DNA. After denaturation of DNA anti-BrdU antibody was added to quantify the incorporated BrdU. This nonradioactive assay does not produce toxic waste as the radioactive assay, thus making it a more widely and practical method to use in laboratories. Moreover, the nonradioactive assay described in our manuscript has better reproducibility and is 20 000 times more sensitive than when radioisotope ^{3}H thymidine was used (Hufnagle *et al.*, 1991).

T-cell response to SEA depends on the expression of TCR V-β and they are different between expressions of TCR V-β subsets between different species of mice that effect the response to SEA between mice strains followed this order: C57BL/6 > AKR/J > BALB/c > C3H/He (Takimoto *et al.*, 1990). In this study we used splenocytes from C57BL/6 mice and are able to detect 20 pg mL^{-1} of SEA without induction by D-galactosamine or lipopolysaccharide. That sensitivity is slightly higher than an assay with human peripheral blood mononuclear cells (PBMC) (0.1 ng mL^{-1}) (Bavari *et al.*, 1995) and 10^{9} times more sensitive than the monkey and kitten bioassay. Moreover, using splenocytes from mice make a more wide and practical method to use than PBMCs, because healthy human donors require approval by the Institutional Review Board and a phlebotomist, which add complexity. Furthermore, using PBMCs from humans is not practical for a clinical method because unlike mice healthy human donors are not always available.

Our results show that the rate of SEA inactivation is dependent on the medium in which the toxin is present.

Autoclaving milk caused reduction of lactose, denaturation of milk protein and a chemical reaction between the amino groups (mainly lysine in casein and lactose), forming a variety of molecules (Van Boekel, 1998). SEA in autoclaved milk inactivated seven times faster than in unautoclaved milk (Fig. 4). This suggests that SEA, in unautoclaved milk, is more thermostable and that undamaged milk has a protective effect on the enterotoxin. Pasteurization is utilized to increase milk safety by destroying spoilage microorganisms and reducing number of viable pathogens, such as *S. aureus*. However, our results show that the enterotoxin will still be present after a longer and higher heat treatment (Fig. 4). This demonstrates a need to prevent early contamination of *S. aureus* in milk. Once the toxin has formed, it is impractical to eliminate it from milk by thermal treatment without compromising the quality of the milk.

The use of an alternative assay instead of the *in vivo* bioassay can dramatically reduce the number of animals used (one mouse spleen can be used for 500 tests). We also discovered that the use of immunomagnetic beads offers several advantages: we can identify specific SEs and concentrate the toxin from a large volume, thus increasing the sensitivity of the assay. T-cell proliferation requires multicell interaction, thus ruling out the proliferation activity that may be caused by food ingredients. In this study we added SEA to food supernatant; we expected that by adding the toxin directly into beef or chicken, the efficiency of the toxin recovery will be lower. Our results show that on concentrating the toxin, as well as removing food matrix interference, we were able to detect 50 ng mL^{-1} of the toxin after incubation of the cells with the beads. By disassociating SEA from the beads, we were able to improve the sensitivity of our assay by 2500 times, and detect 20 pg mL^{-1} of active SEA. The sensitivity of this nonradioactive cell-based assay is 20 000 times more sensitive than radioactivity T-cell proliferation assay (Hufnagle *et al.*, 1991) and 10^9 times more sensitive than the monkey and kitten bioassay.

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